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REMARKS

Claims 22 and 85 are pending. Claim 22 is amended, and the amendment does not constitute new matter.

The Examiner objects to the specification, contending that the title and abstract do not reflect the subject matter of the application as presently claimed, and because the legend to Figure 10 does not identify and describe the two parts of the figure. In response, Applicants have amended the title, the abstract and the legend to Figure 10 to obviate the Examiner's objections. In light of these amendments (which are not new matter), Applicants respectfully request that the Examiner withdraw the objections to the specification.

Applicants also request that the Examiner indicate whether the Information Disclosure Statement and PTO-1449 form mailed by Applicants on December 30, 2003 has now been made of record.

Claims 22 and 85 are rejected under 35 U.S.C. § 101 for lack of utility. Claims 22 and 85 are rejected as failing to meet the written description and enablement requirements of the first paragraph of 35 U.S.C. § 112. For the reasons set forth below, Applicants respectfully traverse the Examiner's rejections of the aforementioned claims and request that the rejections be removed.

I. The Claimed Invention Has Utility

Claims 22 and 85 are rejected under 35 U.S.C. § 101, the Examiner contending that they lack utility. In particular, the Examiner bases the rejection on two grounds: first, that the biological activity of OLD-35 as a polynucleotide phosphorylase ("PNPase") is not adequately supported and, second, that "[t]he specification does not support a substantial utility

regarding the claimed antibodies that bind [OLD-35] for purposes unrelated to the asserted biological activity." With regard to latter, the Examiner further states:

The specification does not disclose a correlation between any specific disorder and an altered level or form of the [OLD]-35 polypeptide wherein an antibody that binds the [OLD]-35 polypeptide has diagnostic utility or utility in a method for inhibiting the activity of the [OLD]-35 protein related to a specific disorder. Also, the specification does not predict whether the claimed [OLD]-35 polypeptide would be overexpressed or underexpressed in a specific, diseased tissue compared to a healthy tissue control.

In a related rejection, claims 22 and 85 are rejected under 35 U.S.C. §112 because, according to the Examiner:

since the claimed invention is not supported by either a substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention without undue experimentation.

Applicants assert that the claims have requisite utility and that a person skilled in the art would know how to use the claimed antibodies, for the following reasons, provided in the order in which the corresponding issues were raised by the Examiner.

First, the claimed antibodies specifically bind to a protein having an amino acid sequence as set forth in SEQ ID NO:42, which constitutes residues 18-713 (truncated due to a sequencing/cloning artifact¹; see Exhibit A, which shows the portion of OLD-35 not represented in SEQ ID NO:42 in pink) of 783-residue OLD-35, a molecule with a recognized biological activity, namely PNPase activity. In a first set of working examples, the specification identifies OLD-35 as a putative PNPase based on sequence homology to a known bacterial PNPase (see Figure 10 and the instant specification at page 36 line 29 through page 37 line 7), and states that

¹ For details, see the Response to Restriction Requirement and Second Preliminary Amendment submitted October 20, 2003.

the Old-35 gene "may play a role in RNA degradation in growth arrested cells" (in the instant specification at page 37, lines 4-7). In a section of the specification that follows, a more definitive statement is made: "[s]ince Old-35 encodes PNPase" (in the specification at page 50, line 26). The Examiner has questioned whether OLD-35 actually has PNPase activity, in view of a number of examples where structure does not correlate with function.

To address the Examiner's concern, Applicants invite the Examiner's attention to the attached publication, Leszczyniecka et al., 2002, "Identification and cloning of human polynucleotide phosphorylase, *hPNPase*^{old-35}, in the context of terminal differentiation and cellular senescence," Proc. Natl. Acad. Sci. 99:16636-16641 ("Leszczyniecka"; attached as Exhibit B), which reports the results of experiments which demonstrate that OLD-35 has PNPase activity *in vitro* (Leszczyniecka at paragraph bridging pages 16638 and 16639, entitled "old-35 is hPNPase"). Given the art recognized relevance of PNPases (for example, in the development of competence in bacteria (a simple model of cell differentiation) and in the differentiation of chloroplasts (in the instant specification at page 49 line 30 through page 50 line 18), the skilled artisan would readily appreciate that an antibody to OLD-35 would have a substantial utility for detecting expression of a biologically important molecule.

Second, irrespective of its biochemical activity, the specification teaches a number of uses for antibodies to OLD-35. The skilled artisan would recognize, and the specification expressly notes (at page 17, lines 17-20), that antibodies directed against "OLD" proteins such as OLD-35 "are useful to detect the expression of OLD proteins in living animals, in humans, or in biological tissues or fluids isolated from animals or humans." The specification further teaches that expression of OLD-35 protein may be used to determine whether a cell is senescent (page

20, lines 28-35), terminally differentiated (page 20, line 37 through page 21, line 6), or growth arrested (page 21, lines 8-15). The skilled artisan would therefore appreciate that an antibody could be used to detect the expression of OLD-35 toward determining whether a cell is senescent, terminally differentiated, or growth arrested, each such use having substantial and credible utility.

Third, contrary to the Examiner's assertion, the specification does teach correlations between changes in the level of Old-35 gene expression and specific disorders. For example, Figure 1 shows that the level of Old-35 mRNA is much higher in senescent Progeria (premature ageing) cell lines (lanes 5 and 6) relative to young fibroblasts (lane 4). Likewise, Figure 2D demonstrates that treatment of melanoma cells with the differentiation-promoting agents interferon beta and mezerein increases the level of Old-35 mRNA. The specification teaches that OLD-35 protein is an alternative to Old-35 mRNA as a means for measuring expression of the Old-35 gene (in the instant specification, for example, at page 20 lines 31-35 and at page 21 lines 3-6 and 11-15). Therefore, the skilled artisan would readily appreciate the utility of antibodies toward OLD-35 protein as a means of detecting differentiation in melanoma cells (a disease) or senescence (a condition) in fibroblasts.

For all the foregoing reasons, the rejection under 35 U.S.C. §101 should be withdrawn. Further, in view of the substantial and credible utility of OLD-35-directed antibodies, and the fact that the skilled artisan knows how to use an antibody to detect a protein, the rejection under 35 U.S.C. §112 for failure to disclose "how to use" the invention should be withdrawn.

II. The Claims Satisfy the Written Description Requirement

Claims 22 and 85 are rejected under the first paragraph of 35 U.S.C. § 112 for failing to comply with the written description requirement. According to the Examiner, the claims as amended contain subject matter which was not described in the specification as originally filed. Specifically, the Examiner contends that addition of the phrase “a protein that is inducible by interferon beta” is new matter because “a protein that is induced by interferon beta” does not appear to be disclosed.

Applicants respectfully disagree with the basis for the rejection. Figure 2B demonstrates that Old -35 mRNA levels are induced by interferon beta. As stated above, the specification teaches that expression of the Old-35 gene may alternatively be measured using mRNA or protein. That Old-35 mRNA is translated into protein is demonstrated by Figure 14 of the specification, which shows localization of a GFP-OLD-35 fusion protein in HeLa cells. Nonetheless, to advance prosecution of the application, Applicants have amended claim 22, without prejudice to subject matter canceled by amendment, to delete the language that has been objected to. Accordingly, the basis for this rejection has been obviated, so that the rejection should be withdrawn.

III. The Claims Are Enabled

Claims 22 and 85 are rejected under the first paragraph of 35 U.S.C. § 112 for lack of enablement. The Examiner contends that the specification does not disclose any particular disease state or condition correlated with altered expression of Old-35 mRNA or Old-35 protein, or provide guidance to assist the skilled artisan on how to make and use antibodies that bind to OLD-35 polypeptide as a diagnostic or therapeutic tool.

Applicants respectfully disagree with the Examiner's position. As discussed in Section I of this paper, with regard to the Examiner's statement that "[t]he specification does not disclose any particular disease state or condition correlated with altered expression of Old-35 mRNA[,]” Applicants maintain that the specification teaches correlations between levels of Old-35 expression (which may be measured alternatively by mRNA or protein expression) and senescence, terminal differentiation, and growth arrest, and with specific instances of melanoma cell differentiation and progeria.

The Examiner questions whether mRNA is validly correlated with protein expression. As stated in Section II above, Figure 14 demonstrates that a nucleic acid comprising Old-35 and GFP was translated to form fluorescent protein. In addition, Applicants invite the Examiner's attention to Leszczyniecka, page 16638, first paragraph, which shows that GST-hPNPase^{OLD-35} having PNPase activity could be expressed.

As for the Examiner's statement that the specification does not “provide guidance to assist the skilled artisan on how to make and use antibodies that bind Old-35 polypeptide as a diagnostic or therapeutic tool[,]” Applicants direct the Examiner's attention to page 17, lines 9-

20, of the specification, wherein guidance to assist the skilled artisan on how to make and use antibodies to Old proteins is explicitly disclosed.


In view of the arguments set forth herein, Applicants respectfully request that the rejection of Claims 22 and 85 under the first paragraph of 35 U.S.C. § 112 for lack of enablement be withdrawn.

CONCLUSIONS

In light of the amendments, Applicants submit that the present application is in condition for allowance of Claims 22 and 85. A Notice of Allowance is therefore respectfully requested.

Respectfully submitted,

BAKER BOTTS L.L.P.



Lisa B. Kole

Patent Office Reg. No. 35,225

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Attachments: Exhibits A and B



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Entrez

PubMed

Nucleotide

Protein

Genome

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PMC

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Details

 ☐ 1: AY027528. Homo sapiens 3'-5...[gi:24943087]

Links

LOCUS AY027528 2615 bp mRNA linear PRI 23-DEC-2002

DEFINITION Homo sapiens 3'-5' RNA exonuclease (OLD35) mRNA, complete cds.

ACCESSION AY027528

VERSION AY027528.1 GI:24943087

KEYWORDS .

SOURCE Homo sapiens (human)

ORGANISM Homo sapiens

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2615)

AUTHORS Leszczyniecka, M., Kang, D.-C., Sarkar, D., Su, Z.-Z., Holmes, M.,
Valerie, K. and Fisher, P.B.TITLE Identification and cloning of human polynucleotide phosphorylase,
hPNPase (old-35), in the context of terminal differentiation and
cellular senescence

JOURNAL Proc. Natl. Acad. Sci. U.S.A. 99 (26), 16636-16641 (2002)

PUBMED 12473748

REFERENCE 2 (bases 1 to 2615)

AUTHORS Leszczyniecka, M., Kang, D.-C. and Fisher, P.B.

TITLE Direct Submission

JOURNAL Submitted (13-FEB-2001) Urology, Columbia University, College of
Physicians and Surgeons, 630 W 168th St, New York, NY 10032, USA

FEATURES Location/Qualifiers

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Identification and cloning of human polynucleotide phosphorylase, *hPNPase*^{old-35}, in the context of terminal differentiation and cellular senescence

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Communicated by Herbert Weissbach, Florida Atlantic University, Boca Raton, FL, October 23, 2002 (received for review August 26, 2002)

Terminal differentiation and cellular senescence display common properties including irreversible growth arrest. To define the molecular and ultimately the biochemical basis of the complex physiological changes associated with terminal differentiation and senescence, an overlapping-pathway screen was used to identify genes displaying coordinated expression as a consequence of both processes. This approach involved screening of a subtracted cDNA library prepared from human melanoma cells induced to terminally differentiate by treatment with fibroblast IFN and mezerein with mRNA derived from senescent human progeria cells. This strategy identified *old-35*, which encodes an evolutionary conserved gene, human polynucleotide phosphorylase (*hPNPase*^{old-35}), that is regulated predominantly by type I IFNs. The *hPNPase*^{OLD-35} protein localizes in the cytoplasm of human cells and induces RNA degradation *in vitro*, as does its purified bacterial protein homologue. Ectopic expression of *hPNPase*^{old-35} in human melanoma cells reduces colony formation, confirming inhibitory activity of this RNA-degradation enzyme. Identification of *hPNPase*^{old-35}, an IFN-inducible 3'-5' RNA exonuclease, provides additional support for a relationship between IFN action and RNA processing and suggests an important role for this gene in growth control associated with terminal differentiation and cellular senescence.

overlapping-pathway screen | terminal cell differentiation | senescent phenotype | interferon-inducible gene | evolutionary conserved gene

Plasticity of the transformed phenotype is suggested by the ability of differentiation-inducing agents to revert the cancerous properties of specific tumors (1–3). This attribute of tumor cells provides the basis for a potentially less toxic form of therapy, “differentiation therapy.” In metastatic human melanoma, a combination of IFN- β and the protein kinase C activator mezerein (MEZ) produces irreversible growth arrest, a loss of tumorigenic competence, and terminal differentiation (1, 4). To define gene-expression changes associated with induction of terminal differentiation, a subtracted cDNA library enriched for genes associated with terminal differentiation was constructed (5). This construction was accomplished by subtracting control HO-1 human melanoma mRNAs from IFN- β + MEZ-treated HO-1 mRNAs, which were temporally collected over a 24-h period (5). This subtracted cDNA library then was screened by random isolation of phage colonies and Northern blotting, high-density cDNA microarray analysis, and reverse Northern screening followed by Northern blotting (5–7). These approaches have identified both unknown and known genes associated with tumor and normal growth control, cell-cycle regulation, IFN response, differentiation, and apoptosis (5–12). Four classes of melanoma differentiation-associated (*mda*) genes have been identified (5, 10).

Terminal cell differentiation and cellular senescence are characterized by changes in cell morphology, lack of responsiveness to mitogenic stimulation, and irreversible growth arrest (1, 4, 13–18).

Normal cells cultured *in vitro* lose their proliferative potential after a finite number of doublings in a process described as cellular senescence (13). Experiments in human diploid fibroblasts and additional cell types document an inverse correlation between replicative senescence and donor age and a direct relationship between replicative senescence and donor-species life span (13, 19, 20). In agreement with this relationship, cells from patients with premature aging syndromes such as Werner's syndrome and progeria achieve a quiescent state more rapidly than normal human fibroblasts (21). Although senescence is a time-dependent process, terminal differentiation can be induced in a variety of cell types by appropriate treatment (2, 3, 16). Growth of HO-1 cells in IFN- β and MEZ results in irreversible growth arrest, altered cellular morphology, modifications in antigenic phenotype, and an increase in melanogenesis (1, 4, 8, 22).

Induction of terminal differentiation in melanocytes by cAMP results in similar and distinct changes in gene expression in comparison with senescent melanocytes (17). Although both pathways result in elevated p21 (WAF1, Cip1, and *mda-6*) expression and an inability to phosphorylate ERK2, only the differentiated cells display elevated levels of p27 and the melanocyte-specific transcription factor MITF (17, 23). Terminal differentiation and senescence also involve additional overlapping gene changes in melanoma and other cell types including enhanced expression of various interleukins (IL-1, IL-15, and *mda-7/IL-24*), cell cycle-regulatory genes [CDK inhibitor, p21 (WAF1, Cip1, and *mda-6*), and DNA damage-inducible genes (GADD153 and GADD34)] (6, 7, 24). Based on these findings, a comparison of senescence and terminal differentiation provides a unique opportunity to identify genes that may govern the growth-suppressive changes underlying both processes.

We have exploited the overlapping-pathway hypothesis as a means of identifying genes coordinately up-regulated during terminal differentiation and cellular senescence. To achieve this goal, a differentiation inducer-treated subtracted HO-1 melanoma cDNA library constructed from temporally spaced poly(A) RNAs from untreated and IFN- β + MEZ-treated HO-1 cells (5) was screened with mRNA from progeria fibroblasts, an accelerated-aging syndrome. This screening stratagem referred to as the overlapping-pathway screen, OPS (Fig. 1), permitted the identification and cloning of *old-35*, human polynucleotide phosphorylase (*hPNPase*^{old-35}), a gene that displays high homology and similar properties with bacterial PNPase, an enzyme involved in RNA

Abbreviations: MEZ, mezerein; *mda*, melanoma differentiation-associated; OPS, overlapping-pathway screen; PNPase, polynucleotide phosphorylase; *hPNPase*^{old-35}, human PNPase (*old-35*).

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY027528).

To whom correspondence should be addressed at: Department of Pathology, Columbia University, College of Physicians and Surgeons, BB-1501, 630 West 168th Street, New York, NY 10032. E-mail: pbf1@columbia.edu.

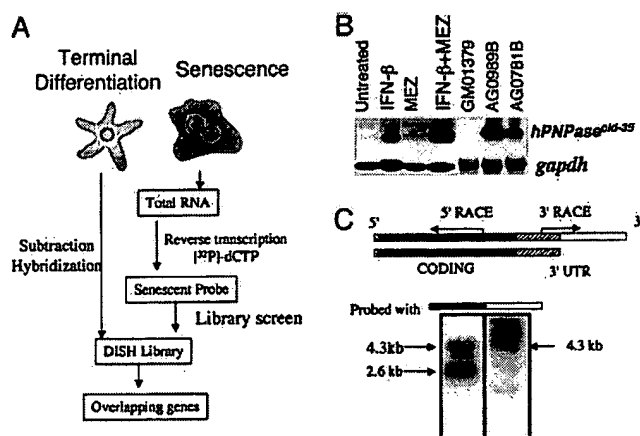


Fig. 1. (A) Schematic representation of the OPS approach. In this application, a differentiation-induction subtraction-hybridization (DISH) library was constructed by subtraction hybridization from terminally differentiated HO-1 human melanoma cells (5). This library was screened with a senescent probe derived from progeria cells. This screening protocol permits the identification of genes displaying parallel (overlapping) changes in expression during terminal differentiation and senescence. (B) Expression of *hPNPase^{old-35}* during terminal differentiation and senescence. Northern blot of *hPNPase^{old-35}* in HO-1 melanoma cells treated for 24 h with IFN-β (2,000 units/ml), MEZ (10 ng/ml) or IFN-β + MEZ (2,000 units/ml + 10 ng/ml), early-passage GM01379 fibroblasts (normal fibroblasts), and two senescent progeroid fibroblasts (AG0781B and AG0989B). *gapdh* was used as a loading control. (C) Representation of two *hPNPase^{old-35}* transcripts visualized in HO-1 cells on Northern blots. The 5' UTR (13 bp) in the 5' region is indicated as a hatched box, the protein-coding region is indicated in black, the 3' UTR common to both *hPNPase^{old-35}* variants is indicated as a hatched box adjacent to the protein-coding region, and the 3' UTR present in the longer *hPNPase^{old-35}* variant is indicated in white. The region between the arrows indicates the EST that was identified by using the OPS approach, and the arrows indicate the directions in which the remainder of the gene was cloned. Northern blot analysis of mRNA from HO-1 cells after IFN-β treatment (2,000 units/ml, 7 h) was performed to determine sizes of the *hPNPase^{old-35}* variants. Two membranes containing the same RNA extracts were probed with either the coding region (black, Left) or the 3' UTR of the longer variant (white, Right).

degradation (25, 26). The present report describes the cloning, expression profile, and biological activity of *hPNPase^{old-35}*, a type I *mda* gene displaying elevated expression in diverse cell types, after treatment with IFN-β and IFN-β + MEZ and exhibiting growth-inhibitory effects in colony-formation assays. In these contexts, *hPNPase^{old-35}* may provide a link between IFN action and growth cessation, which is a defining parameter of terminal differentiation and senescence.

Materials and Methods

Cell Culture. Human melanoma, breast carcinoma, and osteosarcoma cells were obtained from the American Type Culture Collection (ATCC) or as reported and were cultured as described (9, 22, 27, 28). Early-passage human fetal lung fibroblasts (GM01379) and human progeria fibroblasts (AG0989B and AG0781B) (Corriel Repositories, Camden, NJ) were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 units/100 μg/ml) at 37°C in a 5% CO₂/95% air humidified incubator. Sf9 insect cells were cultured in TNM-FH medium (Mediatech Laboratories, Cody, NY) supplemented with 10% FBS and penicillin/streptomycin (100 units/100 μg/ml) at 27°C in a humidified incubator.

Library Screening. AG0989B progeria fibroblast cells were cultured until they became senescent and stained positive for senescence-associated β-galactosidase activity (29). One microgram of poly(A) RNA from AG0989B cells was reverse-

transcribed into radiolabeled cDNA as described (6). A subtracted cDNA library enriched for genes modified during terminal differentiation in human melanoma cells was screened with the senescent cDNA probe as described (5).

RNA Extraction and Northern Blotting. Total RNA was purified from cells by using the RNeasy kit (Qiagen, Valencia, CA). Poly(A) RNA for senescent-probe preparation was extracted by using a Poly(A) Pure kit (Ambion, Austin, TX). For Northern blotting, 10 μg of total RNA was resolved in 1% agarose gels with 2% formaldehyde and transferred to nylon membranes (Hybond-N). The *Xho*I fragment of *hPNPase^{old-35}* cDNA (1.5 kb) and 0.7-kb fragment of *gapdh* were labeled with [α -³²P]dCTP (Roche, Basel). The membrane was hybridized with ³²P-labeled *hPNPase^{old-35}*. The blots were stripped and reprobed with a ³²P-labeled *gapdh* probe and exposed for autoradiography.

Cloning of *hPNPase^{old-35}* and Expression Vector Construction. The full-length *hPNPase^{old-35}* cDNA was cloned from IFN-β-treated HO-1 cell RNA by using C-ORF and 3' RACE in the 5' and the 3' directions, respectively (12). The *hPNPase^{old-35}*-specific primers used in C-ORF and 3' RACE were P1 (5'-TTTGTCTCGT-TTTGATAATG-3'), P2 (5'-TAATGGGAGAACCTATTTCA-3'), and P3 (5'-CTAATTCTCAGTGATTTTTT-3'). The full-length *hPNPase^{old-35}* cDNA was obtained by RT-PCR from IFN-β-treated HO-1 cell RNA using primers P4 (5'-CTAATTC-TCAGTGATTTTTT-3') and P5 (5'-ATTAAACAAATAT-GGGTTAC-3'). The ≈4.3-kb *hPNPase^{old-35}* variant was identified by analysis of the dbEST database and confirmed by acquiring and sequencing ATCC cDNA clone no. 213524. An *hPNPase^{old-35}* expression vector was constructed by cloning an RT-PCR product (5'-GGATCCGCGGCCTGCAGGTACTGC-3' and 5'-GGG-CGCCGCTCACTGAGAAATTAGAT-3') into *Bam*HI- and *Not*I-digested pEF1/His B (Invitrogen). A baculovirus transfer vector, pAcGHLT-*hPNPase^{old-35}*, was constructed by cloning a product amplified by RT-PCR (5'-CGCGGCCCGCGGCCTG-CAGGTACTGC-3' and 5'-GGGCGCCGCTCACTGAGAAATTAGAT-3') into pAcGHLT-B (PharMingen) at the *Not*I site. *hPNPase^{old-35}* RT-PCRred with 5'-GAGCTCAGGATCCGCG-GCCTGCAGGTACTGC-3' and 5'-GGATATCACTGAGAA-TTAGATTGATGA-3' was cloned into the *Sac*I and *Sma*I site of pEGFP-C2 (CLONTECH) to generate GFP-*hPNPase^{old-35}*.

Western Blot Analysis and Fluorescence Microscopy. Ten micrograms of supercoiled plasmid DNA (pEGFP-C2 and pEGFP-C2-*hPNPase^{old-35}*) were transfected into ≈70% confluent HO-1 cells with Superfect (Qiagen) per manufacturer protocol. Two days after transfection, cells were harvested, and protein-sample preparation and Western blotting were performed as described (12). Expression of GFP-*hPNPase^{old-35}* was detected with anti-GFP (CLONTECH) antibody followed by anti-mouse-horseradish peroxidase and ECL (Amersham Pharmacia). For intracellular localization, pEGFP-C2 and pEGFP-C2-*hPNPase^{old-35}* were transfected as described (12) and observed by fluorescent confocal microscopy (×400).

Colony-Forming Assays. Colony-forming assays using HO-1 melanoma cells after transfection with pEF1/His B and pEF1/His B-*hPNPase^{old-35}* were performed as described (12). For adenoviral studies, HO-1 cells were plated at a density of 1 × 10⁵ cells per 6-cm dish, and after 24 h the cells were infected with Ad.*vec*, Ad.*hPNPase^{old-35}*, or Ad.*hPNPase^{old-35}* AS at a multiplicity of infection of 100 plaque-forming units per cell as described (27). Six hours after infection the cells were trypsinized and plated at a density of 10³ cells per 6-cm dish. Colonies ≥50 cells were scored 3 weeks later. The recombinant replication-incompetent adenoviruses were constructed, grown, and assayed as described (30).

Purification of GST-hPNPase^{OLD-35} Fusion Protein and Degradation Assays. GST-hPNPase^{OLD-35} purification was performed by glutathione-Sepharose affinity chromatography as described (12). Purified fusion protein was digested with thrombin (50 units/mg fusion protein) for 2 h at room temperature. After digestion was completed, hPNPase^{OLD-35} protein was separated further on a Sephacryl S-200 column (1.5 × 50 cm) at a constant flow rate of 1.5 ml/min. For enzyme assays, substrate RNA GEM-A0 was prepared as described (31). RNA was diluted 10 times, and 1 μl was used for each degradation-assay reaction. Each reaction was performed in 20 μl of degradation buffer containing 20 mM Tris-HCl (pH 7.5), 1.5 mM DTT, 1 mM MgCl₂, and 20 mM KCl with or without 10 mM Na₂PO₄ for various times as described (32). The reaction products were treated with proteinase K (10 mg/ml) for 30 min at room temperature, and 5 μl of each reaction was spotted on polyethyleneimine cellulose TLC plates (Aldrich) and resolved in 1 M formic acid.

Results

OPS. The OPS approach was used to identify genes mediating altered physiological changes commonly associated with terminal cell differentiation and senescence. Because normal cells senesce slowly in culture, fibroblasts from patients with the accelerated-aging syndrome progeria, which become senescent more rapidly than normal cells, were chosen for this study (33). Progeria cells were subcultured until they exhibited profound morphological changes and senescence-associated β-galactosidase activity, a marker of the senescent state (29). To define genes with expression that changes as a function of induction of terminal differentiation, a subtracted cDNA (differentiation-induction subtraction hybridization) library was prepared from mRNAs isolated over a 24-h period from HO-1 human melanoma cells treated with IFN-β + MEZ (5). This differentiation-induction subtraction-hybridization library was screened with a probe derived from the RNA of senescent progeria fibroblasts (AG0989B) (Fig. 1A). Seventy-five ESTs were identified initially in the OPS screen and designated as *old-1* to *old-75* (data not shown). Among the *old* genes, *old-35* demonstrated an elevated expression pattern associated with both induction of terminal differentiation and senescence and was chosen for further evaluation (Fig. 1B). Expression of *old-35* increased in IFN-β- and IFN-β + MEZ-treated HO-1 cells in comparison with untreated HO-1 controls (Fig. 1B) and in senescent progeroid fibroblasts (AG0781B and AG0989B) relative to early-passage fetal fibroblasts (GM01379) (Fig. 1B). An overlapping expression pattern of *old-35* in senescent fibroblasts and IFN-β + MEZ-induced terminally differentiated human melanoma cells suggests that *old-35* might correlate with or contribute to the cellular changes that characterize both processes.

Cloning of the *old-35* cDNA. The original *old-35* clone obtained from library screening (600-bp) contained an internal region of the *old-35* cDNA and lacked 3'- and 5'-flanking sequences. The 5' and 3' regions of *old-35* were cloned from IFN-β-treated HO-1 cells by using a modified RACE protocol (c-ORF), which resulted in the cloning of a 2,629-bp *old-35* cDNA (ref. 12; Fig. 1C). Northern blotting analysis demonstrated that the *old-35* EST hybridized to two mRNA species of ~4.0 and 2.6 kb in RNA isolated from IFN-β-treated HO-1 cells (Fig. 1C). An ~4-kb *old-35* variant was identified by a BLAST search of the dbEST database, purchased from ATCC (no. 213524), and sequenced. Comparison of the sequences of the two *old-35* cDNA clones (2,629 and 4,331 bp, respectively) indicated identical ORFs that extended from 53 to 2,404 bp, encoding a protein of 783 amino acids with a predicted molecular mass of 86 kDa and a pI of 7.87. The ORF of *old-35* starts at the first AUG codon. Although A⁻³ in the Kozak consensus sequence (AXXaugG) is not conserved, G⁺⁴ is conserved (34). Sequence analysis of *old-35* revealed that this cDNA (~2.6 kb)

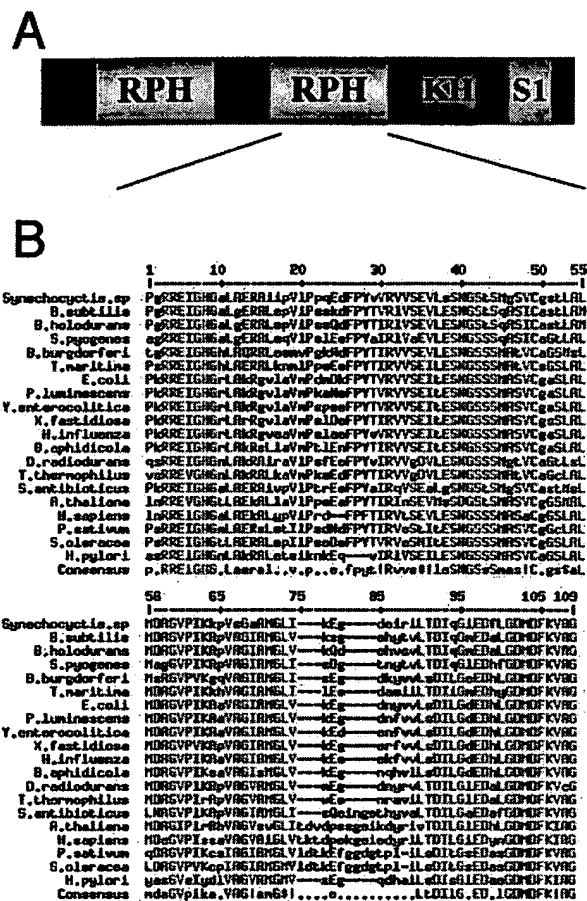


Fig. 2. (A) Structure of PNPase proteins. PNPases have two RNase PH domains, one KH domain, and one S1 domain. (B) Alignment of 20 members of the PNPase family using MultAlin (<http://prodes.toulouse.inra.fr/multalin/multalin.html>, ref. 51). A residue that is highly conserved appears in high-consensus color (90%, red) and as an uppercase letter in the consensus line. A residue that is weakly conserved appears in low-consensus color (50%, blue) and as a lowercase letter in the consensus line. Other residues appear in black. A position with no conserved residue is represented by a dot in the consensus line. I, IV; \$, LM; %, FY; #, NDQE.

contains a less frequently used polyadenylation site (AUUAAA, found in only ~10% of cDNAs) (35). In addition, a canonical polyadenylation site was not detected in the ~4.3-kb variant (Fig. 1A). However, the 4.3-kb clone contained a longer 3' UTR, possibly because of differential polyadenylation. To confirm this possibility, a Northern blot containing total RNA from IFN-β-treated HO-1 cells was probed with either the coding region of the *old-35* gene or the 3' UTR of the longer ~4.3-kb variant (Fig. 1C). Although the ~2.6- and ~4.3-kb bands were detected with a coding-region probe, only the upper ~4.3-kb band was identified with the 3'-UTR probe. Sequence analysis and Northern blot results indicate that the ~4.3-kb mRNA is a variant of *old-35*, which may result from alternative polyadenylation.

***old-35* Is hPNPase.** A BLAST search of the translated sequence of the *old-35* ORF suggested that *old-35* encodes a phosphate-dependent 3'-5' RNA exonuclease-PNPase, previously recognized only in bacteria and plants (36, 37). Further sequence analysis with PROSITE, PFAM, and PRODOM identified four PNPase-specific domains present in the OLD-35 protein sequence (Fig. 2A). Similar to other PNPases, the OLD-35 protein contains two RNase PH domains, one KH domain, and one S1 domain (Fig. 2A). Alignment of the PNPase sequences from a number of different species indicates

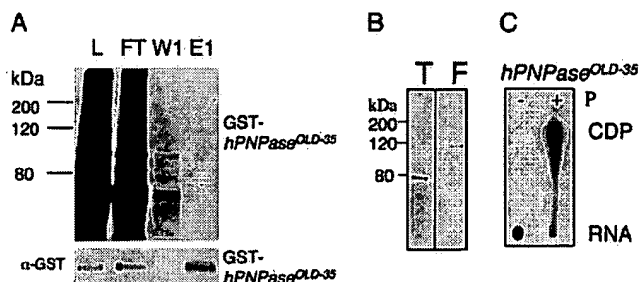


Fig. 3. Purification of GST-hPNPase^{OLD-35}. (A) Coomassie staining of a 10% SDS/PAGE gel. L, lysate; FT, flow through; W1, wash 1; E1, eluate 1. (Lower) The Western blot was probed with anti-GST antibody. (B) Coomassie staining of a 10% SDS/PAGE gel containing thrombin-digested hPNPase^{OLD-35} (T) and undigested GST-hPNPase^{OLD-35} (F). (C) Degradation of RNA by hPNPase^{OLD-35}. The reaction was performed as described in *Materials and Methods* in the absence (–) or presence (+) of phosphate. The degradation products then were resolved on TLC in 1 M formic acid. *E. coli* PNPase (Sigma) was used as a positive control (data not shown).

high conservation in the critical catalytic regions of PNPase, especially in the RNase PH domains (Fig. 2B).

To determine whether sequence conservation translates into conservation of PNPase function (phosphate-dependent degradation of RNA substrates), GST-OLD-35 was expressed by using a baculovirus system and purified to homogeneity by using glutathione affinity chromatography. After purification with glutathione beads, protein purity was assessed by using SDS/PAGE with Coomassie staining and a Western blot with an anti-GST antibody (Fig. 3A). Purified GST-OLD-35 protein migrated as a single 120-kDa band (Fig. 3A). The GST tag was removed by thrombin digestion followed by gel filtration to facilitate functional studies (Fig. 3B). PNPase is a phosphate-dependent 3′–5′ RNA exonuclease that produces nucleotide diphosphates (NDPs) instead of nucleotide monophosphates (NMPs) during the degradation of the RNA substrate (31). Thus, PNPase activity was assayed with or without phosphate with radiolabeled RNA, and undigested radiolabeled RNA and its degradation product (³²P]CDP) were resolved by polyethyleneimine TLC in 1 M formic acid (32). In the absence of phosphate no cytosine diphosphate (CDP) formation was detected, whereas CDP accumulation was observed in the presence of phosphate (Fig. 3C). These data illustrate that OLD-35 protein degraded the RNA substrate in a phosphate-dependent manner. The confirmed phosphate-dependent degradative activity of OLD-35 implies that *old-35* encodes human PNPase as is suggested by sequence homology and indicates that *old-35* is hPNPase^{OLD-35}.

hPNPase^{OLD-35} Is a Type I IFN-Inducible Gene. Northern blot hybridizations were performed to define the induction profile of hPNPase^{OLD-35} after treatment with different IFNs and in different cell backgrounds. hPNPase^{OLD-35} was induced within 3 h by IFN-β (2,000 units/ml) and accumulated until 24 h (Fig. 4A) followed by a gradual decrease in steady-state message level (data not shown). Because IFN-β induces growth suppression in HO-1 cells at 2,000 units/ml, it was important to establish whether up-regulation of hPNPase^{OLD-35} occurs as a result of IFN-induced growth suppression. hPNPase^{OLD-35} expression was induced in HO-1 cells with as little as 1 unit/ml of IFN-β, which is not growth-inhibitory (Fig. 4B), indicating that induction of hPNPase^{OLD-35} expression by IFN can be dissociated from IFN-induced growth suppression. Treatment of HO-1 cells with leukocyte IFN (IFN-α) also resulted in significant up-regulation of hPNPase^{OLD-35} in HO-1 cells, whereas expression of hPNPase^{OLD-35} was marginally stimulated by IFN-γ, and no detectable or consistent induction occurred with tumor necrosis factor α (Fig. 4C). Double-stranded RNA, poly(I)·poly(C), a known inducer

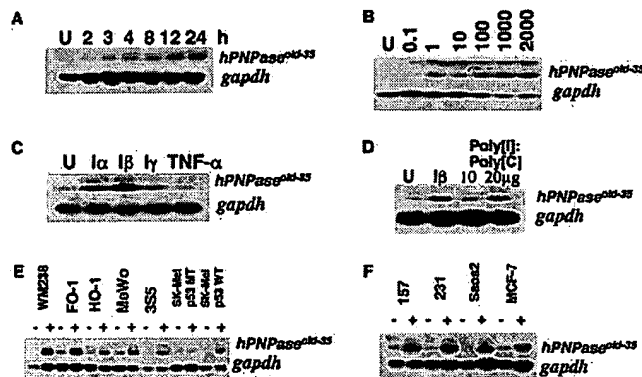


Fig. 4. Induction of hPNPase^{OLD-35} mRNA by IFNs in various cell types. (A) Time-course treatment of HO-1 cells with IFN-β (2,000 units/ml). (B) IFN-β dose response in HO-1 cells. Cells were untreated (U) or treated with 0.1–2,000 units/ml for 7 h. (C) Treatment of HO-1 cells with different cytokines for 17 h. U, untreated; Iα, IFN-α (1,000 units/ml); Iβ, IFN-β (1,000 units/ml); Iγ, IFN-γ (1,000 units/ml); TNF-α, tumor necrosis factor α (10 ng/ml). (D) Effect of IFN-β and poly(I)·poly(C) treatment on hPNPase^{OLD-35} and gapdh expression in HO-1 cells. U, untreated or treated for 24 h with IFN-β (2,000 units/ml), poly(I)·poly(C), 1 × (10 μg/ml), or 2 × (20 μg/ml). (E) Analysis of hPNPase^{OLD-35} and gapdh expression in various human melanomas without (–) or with (+) IFN-β (2,000 units/ml) treatment for 18 h. (F) Analysis of hPNPase^{OLD-35} and gapdh expression in human breast carcinoma (MDA-MB-157, MDA-MB-231, and MCF-7) or human osteosarcoma (Saos2) cells without (–) or with (+) IFN-β (2,000 units/ml) treatment for 18 h. A lower quantity of RNA is present in the untreated versus the treated WM238 sample.

of IFN-α and IFN-β, genes also stimulated hPNPase^{OLD-35} expression (Fig. 4D).

Because hPNPase^{OLD-35} was cloned from HO-1 cells, a metastatic human melanoma cell line, its expression was examined in additional melanoma cell lines. The steady-state *de novo* expression of hPNPase^{OLD-35} was comparable in FO-1, HO-1, MeWo, and 3S5 (a nonmetastatic variant of MeWo) human melanomas with reduced *de novo* expression in the WM238, SK-MEL 110 (mutant p53), and SK-MEL 470 (WT p53) melanoma cell lines (Fig. 4E). However, when treated with IFN-β, expression of hPNPase^{OLD-35} was elevated to variable extents in all the melanoma cell lines. To test for up-regulation of hPNPase^{OLD-35} by IFN-β in cancer cells other than melanoma, MDA-MB-157 (p53-null), MDA-MB-231 (mutant p53), and MCF-7 (WT p53) human breast carcinoma cells and Saos-2 human osteosarcoma cells (p53- and Rb-null) were treated with IFN-β for 18 h, and mRNA levels were determined (Fig. 4F). Treatment of null, mutant, or WT p53 breast carcinoma cells and Saos-2 osteosarcoma cells, which are null for both p53 and Rb, with IFN-β resulted in elevated hPNPase^{OLD-35} expression. Similarly, treatment with IFN-β elevated hPNPase^{OLD-35} expression in normal skin fibroblasts and normal immortal melanocytes, indicating that induction of hPNPase^{OLD-35} by IFN-β is not restricted to cancer cells (data not shown). These experiments document differential regulation of hPNPase^{OLD-35} expression by different cytokines, with type I IFNs (IFN-α/β) being the most active cytokines tested in inducing hPNPase^{OLD-35} expression in HO-1 cells. In addition, expression of WT p53 or Rb is not required for induction of hPNPase^{OLD-35} by IFN-β in melanoma or additional human cancer cell lines.

hPNPase^{OLD-35} Expression Suppresses HO-1 Melanoma Cell Colony Formation. Because growth inhibition is a common event associated with terminal differentiation, senescence, and IFN treatment, it is possible that hPNPase^{OLD-35} could mediate growth inhibition during these processes. To test this possibility initially, the inhibitory effect of hPNPase^{OLD-35} was measured by assaying colony-forming ability of HO-1 cells infected with replication-incompetent adenovirus vectors including Ad.*vec* (an adenovirus lacking the hPNPase^{OLD-35} gene), Ad.hPNPase^{OLD-35}S, or

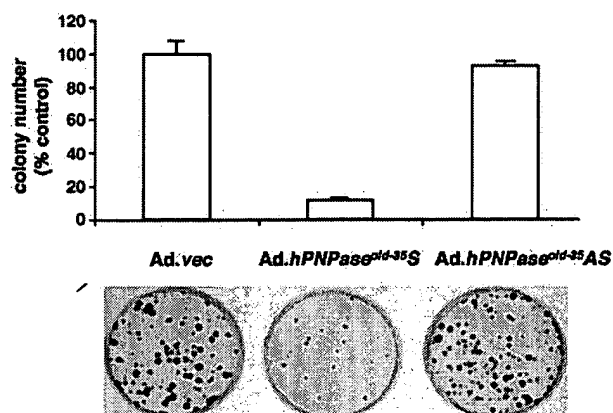


Fig. 5. Colony formation after infection of HO-1 cells with Ad.vec, Ad.hPNPase^{old-35}S, or Ad.hPNPase^{old-35}AS. HO-1 cells (1×10^5) were infected at 100 plaque-forming units per cell; 6 h later cells were reseeded at 10^3 per 6-cm plate, and colony formation was determined 3 weeks later. Graphical representation of three independent experiments using triplicate samples for each condition \pm SD is shown.

Ad.hPNPase^{old-35}AS (Ad.hPNPase^{old-35} antisense). Infection with Ad.hPNPase^{old-35}S resulted in $\approx 90\%$ reduction in colony formation in comparison with infection with either Ad.vec or Ad.hPNPase^{old-35}AS (Fig. 5), confirming that hPNPase^{old-35} has profound growth-suppressing effects in HO-1 cells. The colonies formed in the presence of Ad.hPNPase^{old-35}S were also much smaller than those formed in the presence of either Ad.vec or Ad.hPNPase^{old-35}AS (Fig. 5). To eliminate the possibility that high-level expression resulting from adenovirus infection was the reason for growth-inhibitory properties of hPNPase^{old-35}, transfection experiments with an EF-1 α promoter-driven hPNPase^{old-35} expression vector were performed. This experimental protocol resulted in a significant but decreased reduction in colony formation in comparison with adenoviral infection ($\approx 40\%$, $P < 0.05$, data not shown). Both colony-formation assays document that hPNPase^{old-35} has growth-inhibitory activity, which is consistent with the hypothesis that this gene may contribute to growth modulation during IFN-associated terminal differentiation and senescence. Further studies are required to determine whether growth inhibition is associated with induction of apoptosis or involves reduced cell proliferation.

Cellular Localization of the hPNPase^{OLD-35} Protein. The subcellular localization of hPNPase^{OLD-35} was determined by examining localization of GFP-fusion hPNPase^{OLD-35} by fluorescent microscopy. A Western blot was probed with an anti-GFP antibody to confirm that the fusion protein was expressed in the transfected cell lines. GFP antibody detected proteins of the expected sizes: 30 kDa (GFP) and 120 kDa (hPNPase^{OLD-35}GFP). Fluorescent microscopy of GFP-hPNPase^{OLD-35}-transfected cells demonstrated that hPNPase^{OLD-35} protein localized in the cytoplasm of HO-1 cells, as anticipated for a degradative enzyme (Fig. 6).

Discussion

A phenotype-driven differential gene-cloning method, OPS, has identified hPNPase^{old-35}, which encodes the human homologue of bacterial and plant PNPase. The complete hPNPase^{OLD-35} protein exhibits 37% identity and 54% similarity to the prokaryotic members of this gene family. However, conservation of protein sequence is much higher at specific domain sites where it approaches 70% (Fig. 2B). This sequence conservation also translates into functional conservation. Similar to other PNPses, hPNPase^{old-35} exhibits phosphate-dependent exonuclease activity (Fig. 3C). Although

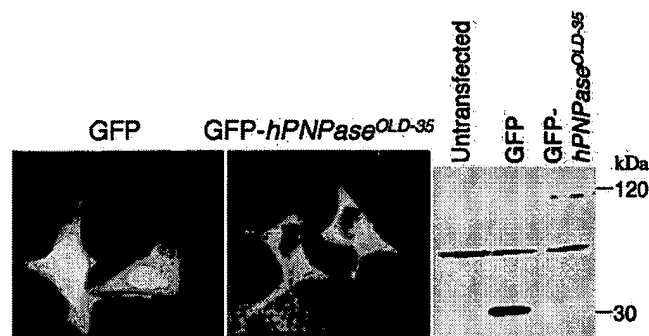


Fig. 6. Cellular localization of hPNPase^{OLD-35}. Cellular localization of hPNPase^{OLD-35} was assessed by using a GFP-hPNPase^{OLD-35} expression plasmid. (Left) HO-1 cells were transiently transfected with a GFP-hPNPase^{OLD-35} construct and observed by fluorescent microscope ($\times 400$). (Right) Western blot of GFP and GFP-hPNPase^{OLD-35}.

polymerization activity associated with bacterial PNPase has not been demonstrated, both sequence analysis and phosphate-dependent exonuclease activity argue that *old-35* is a PNPase, and this article reports the existence of PNPase in the animal kingdom. BLAST searches also indicate the presence of PNPase in *Drosophila melanogaster* and putative PNPase ESTs in *Gallus gallus*, confirming the presence of PNPase in animal cells (data not shown).

PNPase is a component of a multiprotein complex called a degradosome. The bacterial degradosome consists of an endonuclease (RNase E), ATP-dependent helicase (RhlB), an exonuclease (PNPase), and an enolase, a glycolytic enzyme (26, 38). In *Escherichia coli*, decay of mRNAs is initiated by RNase E followed by exonucleolytic degradation at the new 3' ends by PNPase and an additional 3'-5' RNA exonuclease, RNase II (26). Growth of *E. coli* under normal growth conditions is unaffected by deletion of *pnp* (PNPase), but *pnp* mutants exhibit a cold-sensitive growth phenotype preventing growth at temperatures below 30°C (39, 40). In contrast, deletion of both exonucleases, *pnp* and *rnb* (RNase II), is lethal (40, 41). In plants PNPase functions during chloroplast differentiation, most likely as a homohexamer (42, 43), whereas PNPase has not been identified in yeast. However, a multiprotein complex called the exosome functions in yeast in pre-mRNA and mRNA degradation and in rRNA processing (44). The exosome contains endonuclease, 3'-5' exonuclease (RNase PH), and RNA helicase. A human exosome has been described as polymyositis/scleroderma (PM/Scl) overlap syndrome particle, which is related to the yeast exosome (45). The human exosome is reported to contain human homologues of several yeast exosomal components (Rrp40p, Rrp41p, and Rrp46p) and mediates mRNA degradation of AU-rich elements (46–48). Further studies are required to determine whether PNPase is a component of the human exosome, because the structure of this complex is well conserved in evolution.

hPNPase^{old-35} is a type I IFN (α/β)-responsive gene, which is induced as early as 3 h by as little as 1 unit/ml IFN- β in HO-1 melanoma cells (Fig. 4). Moreover, hPNPase^{old-35} is induced by IFN- β in normal and additional cancer cells with diverse genetic backgrounds, suggesting that induction of hPNPase^{old-35} by IFNs represents a general cellular response to these cytokines. The fact that double-stranded RNA, which mimics viral replication intermediates, stimulates hPNPase^{old-35} expression suggests a possible role of hPNPase^{old-35} in IFN-mediated antiviral responses. Overexpression of hPNPase^{old-35} in human melanoma cells results in growth suppression (Fig. 5). In these contexts, hPNPase^{old-35} may play a pivotal role in IFN-mediated antiviral response by modulating cell growth.

It is established that IFNs induce a plethora of genes, many of which function in mRNA stability and translation (12, 49, 50).

Two well characterized pathways are translational repression by double-stranded RNA-dependent protein kinase (PKR) and RNA degradation by 2–5 A-dependent RNase L (OAS/RNase L) (49). *hPNPase^{old-35}*, *mda-5* (a putative RNA helicase with growth-suppressive activity) (12) and RNase II (*mda-E-63*) (24) were identified as IFN-inducible genes in human melanoma cells, suggesting the existence of alternative IFN-stimulated RNA-decay pathways in mammalian cells. It remains to be determined how IFNs acting through these molecules may modify gene expression by regulating RNA degradation/stability. However, based on induction in response to IFN treatment or viral infection and cytoplasmic localization, it is possible that an exosomal structure consisting of *hPNPase^{old-35}* and *mda-5* may be assembled in concert with other proteins that then target growth-related mRNA species for degradation. In future studies it will be essential to determine the specificity of *hPNPase^{old-35}* action, this information will permit elucidation of the mechanism by which *hPNPase^{old-35}* regulates cell growth during IFN treatment, differentiation, and senescence.

A cloning strategy called OPS has been developed to facilitate the identification of genes displaying overlapping expression profiles as a function of induction of complex analogous phenotypic changes in target cells. Presently, OPS was applied to the processes of terminal differentiation and cellular senescence resulting in cloning of the human homologue of PNPase, *hPNPase^{old-35}*. As predicted based on the underlying premise of OPS, steady-state levels of *hPNPase^{old-35}* message were higher in senescing cells in comparison with proliferating fibroblasts and were also increased during terminal cell differentiation. Because OPS is a phenotype-driven screening methodology and ectopic expression of *hPNPase^{old-35}* is growth-suppressive, it seems that this gene con-

tributes toward the common attribute of growth arrest that is shared between the processes of terminal differentiation and senescence. Preliminary studies suggest that expression of antisense *hPNPase^{old-35}* in HO-1 human melanoma cells inhibits IFN- β + MEZ-induced terminal differentiation, indicating that *hPNPase^{old-35}* is essential for induction of this process (data not shown). *hPNPase^{old-35}* encodes a putative 3'–5' RNA exonuclease, suggesting that it might regulate the stability of downstream genes involved in terminal cell differentiation. Although the specific targets of *hPNPase^{old-35}* action are not known currently, they might involve important regulatory proteins involved in signaling such as *c-fos*, *c-myc*, or *c-jun*. Because these molecules have been found to participate in programs of proliferation and differentiation, where they are regulated at the level of mRNA stability, it is likely that they can be modulated by *hPNPase^{old-35}*.

As a final cautionary note, although quite robust in identifying numerous interesting and potentially relevant genes involved in growth control, differentiation, and cancer suppression, previous screening approaches did not identify *hPNPase^{old-35}* in the context of differentiating melanoma cells (5–7, 12, 24). This suggests that multiple cloning approaches will be required to define the full spectrum of differentially regulated genes associated with and contributing to growth control and terminal differentiation in cancer cells.

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